REVIEW

Extended-spectrum β-lactamases: structure and kinetic mechanism

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ABSTRACT

Recent years have seen an explosion in the numbers of extented spectrum class A β-lactamases (ESBLs). The steady-state kinetic parameters for hydrolysis of β-lactams by ESBLs is discussed in the light of what is known about the structure of these mutant enzymes.

Keywords β-lactamase, β-lactamase inhibitior, conformation change, ESBL, kinetics, review.

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INTRODUCTION

Recent years have seen the rapid increase in plasmid-encoded class A β-lactamases in clinical populations of pathogenic Gram-negative bacteria (Fig. 1). Of particular importance are variants of the broad-spectrum β-lactamases derived from TEM-1, TEM-2 and SHV-1 that have acquired mutations that extend their substrate specificity to include the advanced-generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. The focus of this review will be to examine what is known about the way in which the mutations enable the hydrolysis of these important antimicrobial agents.

KINETIC MECHANISMS

Before examining the mutations that have been identified in extended-spectrum β-lactamases (ESBLs), it is worthwhile considering what effects mutation might be expected to have on the observable steady-state kinetic parameters. It is reasonable to suppose that the consequence of a spectrum-expanding point mutation will be to change one or more of the microscopic rate constants that describe the catalytic mechanism. Such alterations will be manifest in the steady-state kinetic parameters that, as shown in Fig. 2, are composite terms involving different combinations of the microscopic rate constants.

The turnover number, k_{cat}, depends on the rates of interconversion of the intermediates, especially the rates of formation and hydrolysis of the acyl–enzyme complex. Therefore, it is the best parameter to describe the hydrolytic activity of β-lactamases. This steady-state kinetic parameter will serve to identify any mutations that affect the reactivity of the free enzyme towards β-lactams, as well as mutations that affect the hydrolysis step. It does not, however, serve to identify mutations that affect the recognition of substrates by the enzyme.

The apparent affinity or half-saturation constant, K_M, is frequently referred to as the Michaelis constant because of its equivalence to the half-saturation constant defined for the Michaelis–Menten mechanism (which does not, however, include a covalent intermediate). The K_M includes all the rate constants describing the catalytic steps of β-lactam hydrolysis. Hence, it is the steady-state kinetic parameter that should be most sensitive to the effects of mutations, and is the parameter best suited to the identification of extended-spectrum mutants.

The apparent second-order rate constant, k_{cat}/K_M, has also been used as a basis to characterise ESBLs and is frequently called 'catalytic efficiency'. However, as can be seen in Fig. 2, this constant depends only on the rate constants involved in the formation of the acyl–enzyme intermediate, and none of the steps involved in hydrolysis contribute. It is therefore inappropriate

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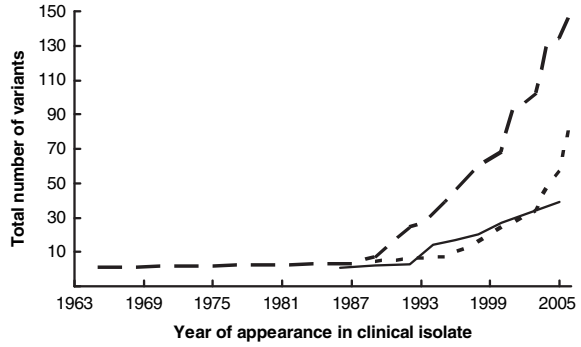


Fig. 1. Increasing incidence of extended-spectrum class A β-lactamases. The cumulative total number of extended-spectrum variants of TEM (dashed line), SHV (dotted line) and CTX-M (solid line) series is shown as a function of the year in which the isolates were first reported. Data compiled from http://www.lahey.org/studies/webt.htm

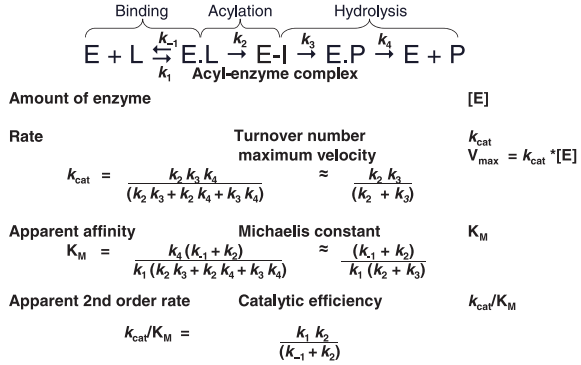


Fig. 2. A simple mechanism for hydrolysis.

to call this parameter 'catalytic efficiency'. k_{cat}/K_M is independent of the hydrolytic activity of the enzyme, and no mutations that affect the rate of hydrolysis of the ester or release of hydrolysed product will change this parameter. Mutations that affect the recognition of substrate by the enzyme, or the reactivity of the enzyme towards β-lactams, will affect k_{cat}/K_M.

The effects of mutations that increase the ability to hydrolyse a particular substrate are, mostly, likely to be manifest as: (1) the association rate (k_1) increases; (2) the dissociation rate (k_1) decreases; (3) the acylation rate (k_2) increases; and (4) the hydrolysis rate (k_3) increases. The effects of such changes in the microscopic rate constants on the steady-state kinetic parameters are summarised in Table 1. Significant change in k_{cat} (>ten-fold) can only occur if k_2 and k_3 are both altered; significant changes in K_M could result from changes in the association and dissociation rates or the hydrolysis rate, while large changes in k_{cat}/K_M should only be expected as a result of an increased rate of association.

BRANCHED PATHWAYS AND CONFORMATIONAL CHANGE

Experience has shown that the simple mechanism discussed above is frequently inadequate to describe the kinetic mechanism of β-lactamases. The most frequently invoked causes of departure from the simple mechanism are branched pathways, in which partitioning between parallel reaction pathways may occur [1]. The branch may be the result of a chemical rearrangement of the substrate triggered by the attack of the enzyme on the β-lactam moiety, or it might result from a change in the conformation of the protein triggered by binding of, or reaction with, the β-lactam. It is quite probable that both events may occur in some reactions. Two simple cases (Fig. 3) are considered below.

Table 1. Changes in steady-state kinetic parameters as a consequence of individual changes in rate constants

Change in microscopic rate constant	Effect on steady-state kinetic parameter		
	k_{cat}	K_M	k_{cat}/K_M
K_1 increases	No effect	Decreases proportionately	Increases proportionately
K_{-1} decreases	No effect	Decreases proportionately	Reaches limiting value when $k_2 > k_{-1}$
K_2 increases	Reaches limiting value when $k_2 > k_3$	Reaches limiting value when $k_2 > k_3$	Reaches limiting value when $k_2 > k_{-1}$
K_3 increases	Reaches limiting value when $k_3 > k_2$	Decreases proportionately	No effect
K_2 and k_3 increase	Increases proportionately	Reaches limiting value when $k_2 > k_{-1}$	Reaches limiting value when $k_2 > k_{-1}$

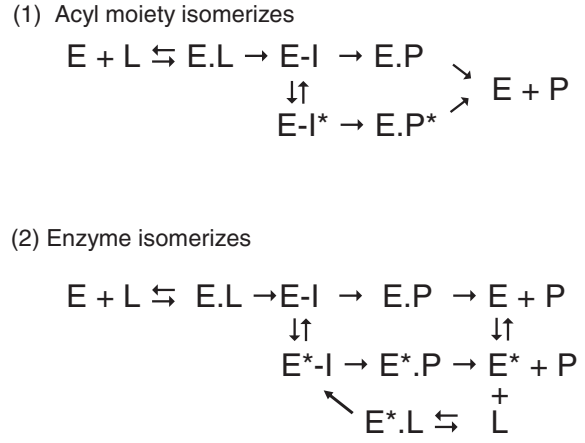


Fig. 3. Branched pathways.

Chemical rearrangement of the acyl moiety

Chemical rearrangement of the acyl moiety derived from the substrate has been described for several cephalosporins and for compounds known as β-lactamase inhibitors (often poor substrates) [1].

Formally, the rearrangement can be considered to occur at the first acyl–enzyme intermediate and to generate a second acyl–enzyme species that is more slowly hydrolysed than the first (Scheme 1, Fig. 3). In this mechanism, the turnover number, k_{cat}, reflects the acylation rate, the rates of hydrolysis of the individual acyl–enzymes and the rate of their interconversion (k_5). If the isomerisation reaction is significantly faster than the rate of hydrolysis of the un-isomerised acyl–enzyme, then k_{cat} will depend only on the acylation rate and the hydrolysis rate of the isomerised complex.

Chemically, the rearrangements typically include loss of a proton from the nitrogen atom that was formerly part of the β-lactam ring through ring-opening and double-bond formation (Fig. 4). This species may go on to form further intermediates (see below), but loss of the proton appears to be sufficient to render this species relatively inert for class A β-lactamases [1].

Mutations can affect the course of branched pathways in several ways. For example, those mutations that change charged residues (e.g., the Glu104Lys mutation in TEM-3) will alter the dielectric constant and hydrogen-bonding patterns in the active site region where the chemical rearrangement is taking place. This could very strongly affect the rates of proton transfer and charge separation in any of the rearrangements. This, in turn, could allow new branch points, or could account for a branch point being passed by if the reaction constituting the branch becomes relatively slow.

Conformational change in the enzyme

Conformational change in the protein during its reaction with substrates has been postulated for a number of β-lactamases. Indirect experimental evidence of significant changes in protein conformation has been obtained from reactions in solution, particularly from spectroscopic and stability measurements [1,2]. For example, extensive hydrogen-to-deuterium exchange with the deuterated solvent during the deacylation reaction of TEM-3 was detected by time-resolved Fourier-transform infrared spectroscopic studies [3], suggesting that there must be a substantial structural change during the deacylation process that allows access to the core of the protein. As yet, there is little evidence of such changes from X-ray crystallographic studies of reactions occurring with the crystallised protein, or from proteins crystallised after reaction with inhibitors [4–6]. This dichotomy leads to an inadequacy in describing the mechanism of β-lactamases and necessitates further investigation of these reactions using techniques such as nuclear magnetic resonance [7] and infrared spectroscopy [3], which can generate time-resolved structural information from solution reactions.

The consequences of protein conformational change will be manifest in the steady-state kinetic parameters according to the step at which the change occurs (Fig. 3). The most probable steps with which conformation change might be associated are the formation of the first acyl–enzyme intermediate and the breakdown of the enzyme–product complex. Isomerisation of the free enzyme, e.g., between less active and more active states, has also been described [8]. If only the bound species exist in two (or more) conformations, and the enzyme relaxes to ground-state upon release of product, the steady-state kinetic parameters will show the same dependency on the microscopic rate constants as discussed above for the case where the acyl moiety undergoes chemical rearrangement. On the other hand, if the

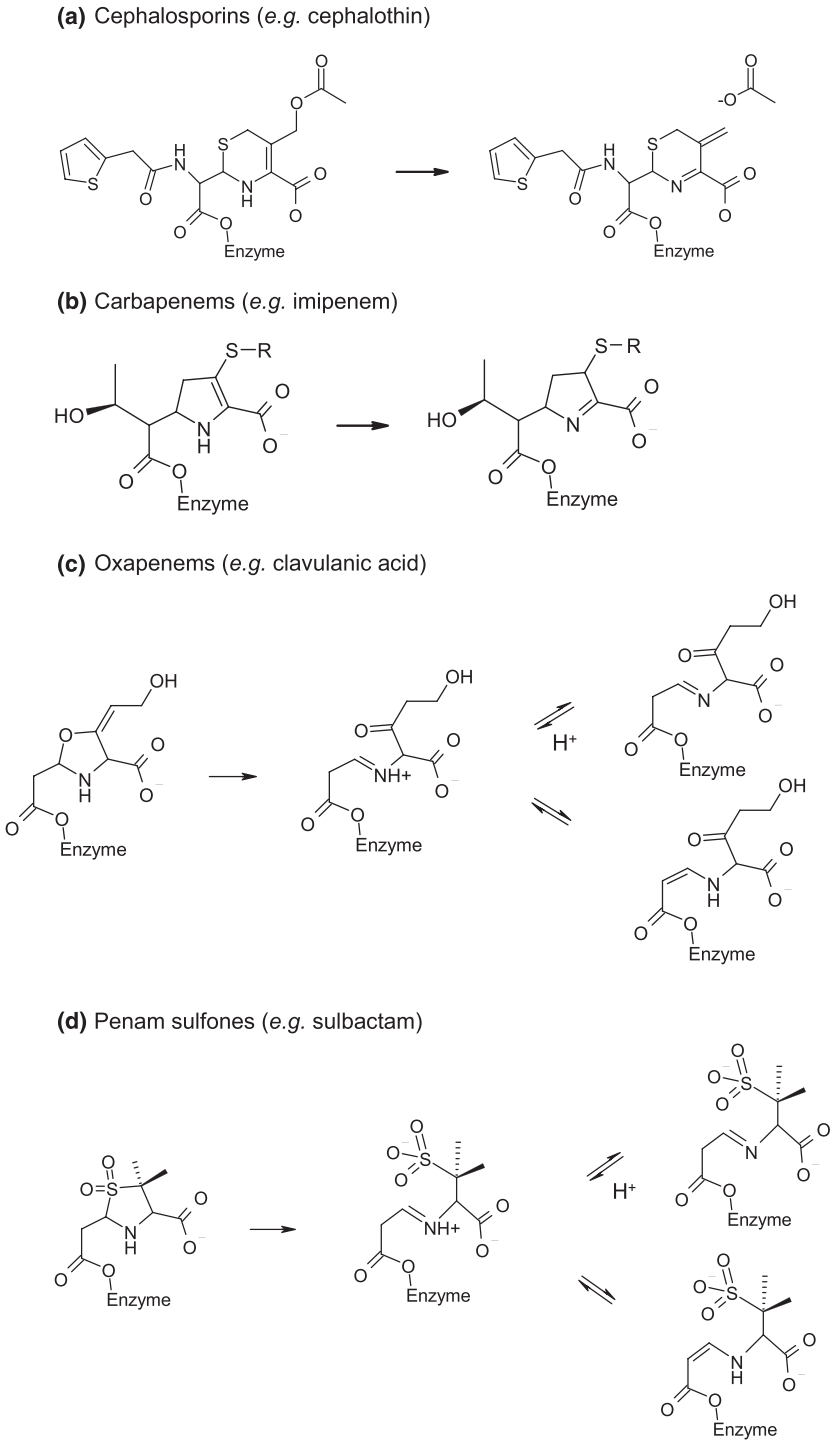


Fig. 4. Examples of rearrangement of the acyl moiety during reaction of β-lactams with β-lactamases. (a) Cephalosporins (e.g., cephalothin). (b) Carbapenems (e.g., imipenem). (c) Oxapenems (e.g., clavulanic acid). (d) Penam sulfones (e.g., sulbactam).

free species of the enzyme also undergoes isomerisation, the possibility of parallel catalytic cycles exists, and the reaction may proceed through multiple phases as it settles down to a steady state in which the partition between the parallel cycles is determined by the relative magnitude of the respective rate constants for catalysis and the isomerisation rates [8].

Mutations that affect the flexibility of the protein may have consequences for the reaction

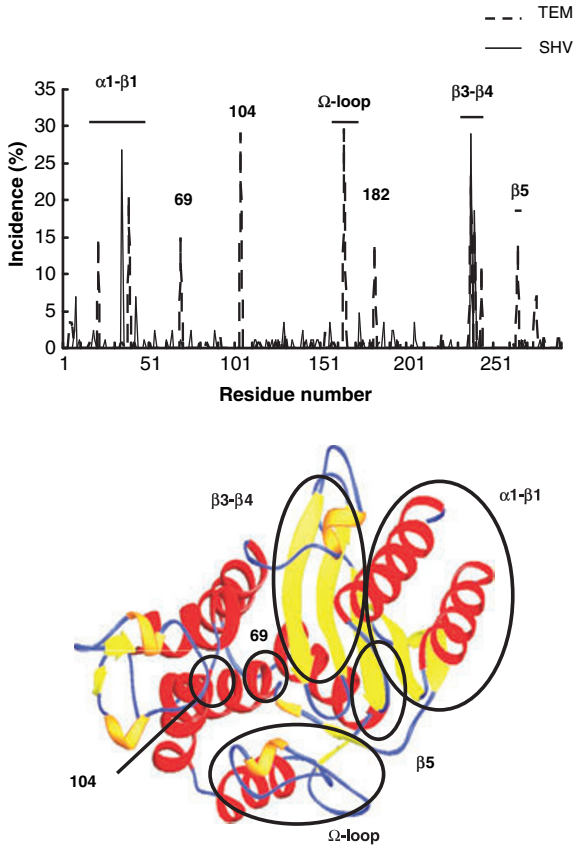
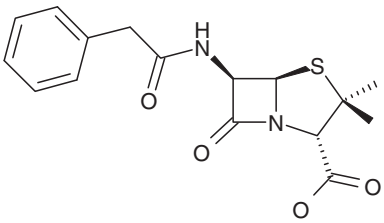


Fig. 5. Frequent loci for substitutions affecting the substrate specificity of class A β-lactamases.

pathway if they are involved in the substrate-induced conformational change.

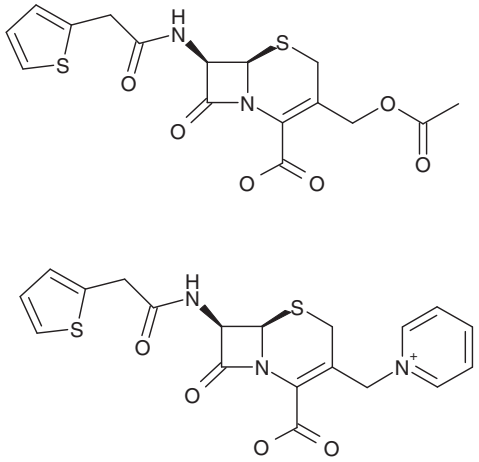
SUBSTRATES AND INHIBITORS

Benzylpenicillin



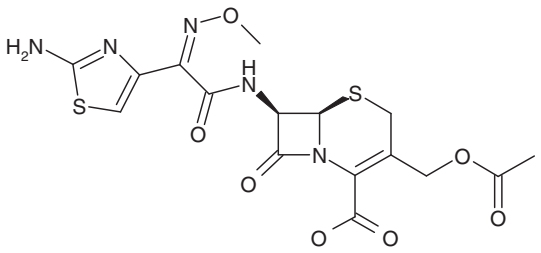
Benzylpenicillin is rapidly hydrolysed by most class A β-lactamases and exhibits simple, linear kinetics where analysis has been undertaken. Although chemical rearrangement consequent upon opening of the thiazolidine ring would be possible [9], this does not appear to be of any significance in the enzymes under consideration.

Cephalothin and cephaloridine



Cephalothin and cephaloridin, both of which are first-generation cephalosporins, react rapidly with class A β-lactamases and are readily hydrolysed by most. Both have potential leaving groups in the 3′-substituents, making possible the elimination and chemical rearrangement (Fig. 3a) that occurs during the reaction with the enzyme [10,11]. Branched pathways with transient inhibition or non-linear kinetics may therefore be observed with some enzymes. The acetoxy substituent of cephalothin is a better leaving group than the pyridinium of cephaloridine, and the elimination may be nearly concomitant with acylation for the former compound, whereas the β-lactam hydrolysis product still bearing the 3′-leaving group may be released into solution during the reaction of cephaloridine with some enzymes [10]. Thus, interpretation of changes in kinetic parameters for the hydrolysis of these cephalosporins, and cephaloridine in particular, has to be cautious.

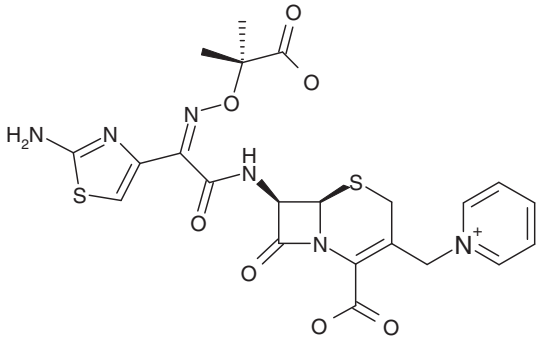
Cefotaxime



Cefotaxime was one of the earliest third-generation cephalosporins, which are characterised by the oxyimino acyl side chain. Compared to the

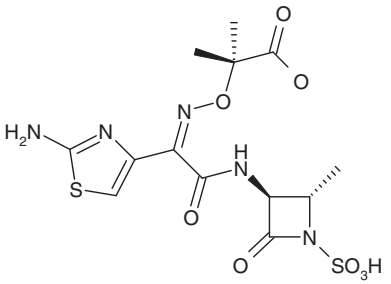
side chains of benzylpenicillin and the first-generation cephalosporins, the oxyimino side chain is large and inflexible, making it more difficult to accommodate in the active site of class A β-lactamases. As discussed below, many of the substitutions found in ESBLs are thought to help ameliorate this poor fit. Cefotaxime has the same 3′-substituent as cephalothin and is therefore conveniently compared with the latter when examining the effects of mutations on kinetics.

Ceftazidime



Ceftazidime is also a third-generation cephalosporin. It has the same 3′-substituent as cephaloridine and is best compared with this compound when examining the effects of mutations. The oxyimino group has an additional negative charge that would normally be repelled by acidic side chains in the periphery of the class A active site (Glu104, Glu240), and several of the mutants with enhanced activity towards ceftazidime have lysine substituted for glutamate at one or both of these positions.

Aztreonam



Aztreonam is a monobactam with the same acylamino side chain as ceftazidime. It has little propensity towards chemical rearrangement and is therefore convenient for checking whether mutations affect only the side chain interactions or have a wider influence on the branching pathways open to ceftazidime.

MUTATIONS LEADING TO CHANGES IN SUBSTRATE SPECIFICITY OF CLASS A β-LACTAMASES

Hot spots in class A variants

Substitutions at 87 loci have been identified in class A β-lactamases belonging to the TEM, SHV and CTX-M series. There is a considerable spread in the incidence of such substitutions (Fig. 5) and, of course, not all affect the kinetic properties of the enzymes. The most frequently occurring mutations cluster in several groups: in the α1–β1 strands, in the Ω-loop, in the β3–β4 strands and in the β5 strand. In addition, there are highly prevalent point mutations at positions 69, 104 and 182 in enzymes of the TEM series.

Table 2. Kinetic parameters for hydrolysis of substrates by TEM-109 (Met69Leu, Glu104Lys, Arg164His) and TEM-6 (Glu104Lys, Arg164His)

	Kinetic parameters					
	k_{cat} (/s)		K_M (μM)		k_{cat}/K_M (/mM/S)	
Compound	TEM-6	TEM-109 (CF)	TEM-6	TEM-109 (CF)	TEM-6	TEM-109 (CF)
1	134	171 (0.28)	10	20 (1)	13	8 (−0.6)
2	74	15 (−3.9)	247	181 (−0.4)	0.3	0.1 (−2)
4	9	21 (1.3)	71	330 (3.6)	0.1	0.1 (0)
5	105	56 (−0.9)	247	226 (−0.1)	0.4	0.2 (−1)
6	22	38 (0.7)	51	130 (−1.5)	0.3	0.4 (0.3)

Data taken from Robin *et al.* [21]. CF indicates the change factor, calculated as CF = (parameter of mutant/parameter of 'parent') − 1, when the value of the parameter of the mutant is greater than that of the 'parent', and as CF = 1 − (parameter of 'parent'/parameter of mutant), when the value of the parameter of the mutant is less than that of the 'parent'.

Table 3. Kinetic parameters for hydrolysis of substrates by TEM-1 and a mutant with lysine at position 104

	Kinetic parameters					
	k_{cat} (/s)		K_M (μM)		k_{cat}/K_M (/mM/s)	
Compound	E104	K104 (CF)	E104	K104 (CF)	E104	K104 (CF)
1	1100	840 (−0.32)	24	21 (−0.14)	45 000	40 000 (−0.12)
2	150	150 (0)	260	240 (−0.08)	600	620 (0)
3	1000	670 (−0.5)	370	600 (0.62)	2700	1100 (−1.4)
4	2	25 (12.5)	1100	1000 (−0.1)	1.8	25 (13)
5	0.02	0.3 (14)	300	80 (−2.8)	0.066	3.7 (56)
6	0.2	2.5 (11.5)	200	160 (−0.25)	1	16 (15)

Data taken from Petit *et al.* [22]. CF indicates the change factor, calculated as CF = (parameter of mutant/parameter of 'parent') − 1, when the value of the parameter of the mutant is greater than that of the 'parent', and as CF = 1 − (parameter of 'parent'/parameter of mutant), when the value of the parameter of the mutant is less than that of the 'parent'.

Substitutions in the α1–β1 region

Substitutions in the α1–β1 region include those at position 21 (TEM-4 and derivatives, TEM-67), position 35 (SHV2A and derivatives, TEM-130), position 39 (TEM-2 and derivatives) and position 43 (SHV-7 and derivatives). As yet, no substitution in this region has been shown to have a significant effect on the steady-state kinetic mechanism. The substitutions may effect secretion of the pre-protein, folding kinetics, or stability of the folded protein (e.g., the T_m of TEM-2 is 54.7°C compared to 51.5 °C for TEM-1) [12].

Substitutions at position 69

Substitutions at position 69 are associated with an inhibitor-resistant phenotype when present as a single substitution (e.g., in TEM-33 or SHV-49) but are also found in combination with substitutions associated with an extended-spectrum phenotype (e.g., TEM-35). The substitutions replace methionine with an aliphatic amino-acid (valine, isoleucine or leucine) and generally result in decreased k_{cat} and a lower k_{cat}/K_M (Table 2). The decrease in reactivity of the enzyme is beneficial in slowing down the reaction with inhibitors such as clavulanic acid, altering the balance between acylation and rearrangement along the complex reaction pathways that ensue after acylation.

X-ray crystallography suggests that the substitutions of methionine affect the position of Ser70 or distort the active site, causing misalignment of Ser70 and Ser130 [5]. These alterations in local structure of critical catalytic residues are consistent with the decreased turnover and lower apparent affinity of the mutant enzyme.

Substitution at position 104

The glutamate-to-lysine change at position 104 is one of the commonest substitutions in the TEM series, being found in 41 variants. It is rare as a single mutation but is frequently combined with substitutions at positions 164 (19 variants), 239 (19 variants) and 182 (12 variants). It has also been found in combination with substitutions at positions 237 (five variants), 265 (five variants) and 240 (three variants). The substitution has not yet been found in natural isolates of SHV but site-directed mutants have been studied [13].

The mutation has a slightly negative effect on the kinetic parameters for hydrolysis of the rapidly hydrolysed substrates **1–3** but results in increased values of k_{cat} for β-lactams with oxyimino side chains **4–6** (Table 3). The mutation also produces a significant change in K_M for ceftazidime, which has been attributed to interaction between the negatively charged side chain of the β-lactam and the new positive charge at position 104. However, there must be other factors involved, as the K_M of the mutant for aztreonam, which has the same side chain as ceftazidime, does not change very much. The pattern of changes in kinetic parameters strongly suggests a selective increase in the association rate (k_1 in Fig. 1) and in the rates of acylation and deacylation (k_2 and k_3) for substrates **4–6**. This would be consistent with widening of the active site to admit the larger oxyimino side chain (affects k_1) and to specifically improve its orientation with

Table 4. Kinetic parameters for hydrolysis of substrates by TEM-7 (Arg164) and TEM-1

	Kinetic parameters					
	k_{cat} (/s)		K_M (μM)		k_{cat}/K_M (/mM/s)	
Compound	TEM-1	TEM-7 (CF)	TEM-1	TEM-7 (CF)	TEM-1	TEM-7 (CF)
1	1600	40 (−39)	19	3.1 (−5)	84 000	13 000 (−5.5)
2	160	20 (−7)	246	80 (−2)	650	250 (−1.6)
3	1500	26 (−58)	682	87 (−6.8)	2200	300 (−6.3)
4	9	1.5 (−5)	6000	100 (−59)	1.5	15 (9)
5	0.3	9 (30)	4300	1000 (−3.3)	0.07	9 (2300)
6	1	4 (3)	1430	1333 (0.1)	0.7	3 (3.2)

Data taken from Raquet *et al.* [23]. CF indicates the change factor, calculated as CF = (parameter of mutant/parameter of 'parent') − 1, when the value of the parameter of the mutant is greater than that of the 'parent', and as CF = 1 − (parameter of 'parent'/parameter of mutant), when the value of the parameter of the mutant is less than that of the 'parent'.

respect to the catalytic centre, so that attack on the β-lactam ring (k_2) is more efficient. The improved orientation would also appear to be preserved in the acyl–enzyme complex, such that its hydrolysis (k_3) is also enhanced.

Substitutions in the Ω-loop

Substitutions in the Ω-loop are the commonest in the TEM series, with at least 42 derivatives known with replacements at Arg164. The residue is mutated to serine (23 variants), histidine (16 variants) and rarely cysteine (three variants). It may be the only mutation present in a variant that affects the kinetic mechanism (TEM-7, -12, -29, -53, -115 and -143) or, more commonly, it is found in combination with other substitutions that are known to also modify the kinetics. These include substitutions at positions 104 (19 variants), 237 (eight variants), 238 (three variants), 240 (14 variants) and 265 (seven variants). The combination of substitutions at positions 164 and 238 does appear to be very favourable, as only three of the 33 variants with the substitution at position 238 also have a substitution at position 104.

The substitutions significantly impair the hydrolysis of substrates **1–3**, with large decreases in k_{cat} and K_M resulting in five-to ten-fold decreases in k_{cat}/K_M (Table 4). In contrast, k_{cat}/K_M for the hydrolysis of oxyimino cephalosporins is increased more than five-fold. For cefotaxime (**4**), this is the result of an increase in apparent affinity (K_M is significantly lower), whereas the apparent affinity decreases but the turnover number is greatly increased for ceftazidime and, to a lesser extent, aztreonam, (Table 4). The pattern of changes strongly suggests that the association rate is increased for all substrates but, while the mutation improves the alignment of the oxyimino substrates, this is at the expense of alignment of the substrates with small side chains (**1–3**), for which the hydrolysis rate drops markedly.

X-ray crystallography of mutants with this substitution suggests that the pattern of hydrogen bonding in the binding pocket that receives the acylamino side chain is altered in the mutants. This could explain why the consequences of this mutation appear to be manifest in different ways, according to the acylamino side chain of the substrate.

Substitution at position 182

Substitution at position 182, in which methionine is replaced with threonine (19 times) or isoleucine (once), is only found in the TEM series. It is relatively more common among TEM-1 derivatives (16%) than among TEM-2 derivatives (6%). It has only minor consequences for the kinetics of the enzymes in which it is found (changes in any of the parameters are usually less than five-fold [6,14]), and the mutant occurs as a natural variant (TEM-135) that has properties similar to TEM-1 [15]. The substitution affects the dynamics of folding of nascent protein [14,16] or the thermal stability of the folded protein [6], and has therefore been suggested to ameliorate the deleterious effects of other mutations on expression of β-lactamase activity [6,14,16,17]. It is frequently found in combination with substitutions that have little deleterious effect on thermal stability of the folded

Table 5. Kinetic parameters for hydrolysis of substrates by TEM-24 (39Lys, Glu104Lys, Arg164Ser, Ala237Thr, Glu240Lys) and TEM-46 (39Lys, Glu104Lys, Arg164Ser, Glu240Lys)

	Kinetic parameters					
	V_{max} relative to hydrolysis of 1		K_M (μM)		V_{max}/K_M relative to hydrolysis of 1	
Compound	**TEM-46**	**TEM-24 (CF)**	**TEM-46**	**TEM-24 (CF)**	**TEM-46**	**TEM-24 (CF)**
2	0.46	2.8 (5.1)	100	43 (−1.3)	0.02	0.36 (17)
4	0.13	1.3 (9.0)	50	50 (0)	0.01	0.14 (13)
5	0.19	14 (73)	159	380 (1.4)	0.06	0.21 (2.5)
6	0.35	1.2 (2.4)	12	42 (2.5)	0.09	0.17 (0.89)

Data taken from Chanal-Claris *et al.* [24]. CF indicates the change factor, calculated as CF = (parameter of mutant/parameter of 'parent') – 1, when the value of the parameter of the mutant is greater than that of the 'parent', and as CF = 1 – (parameter of 'parent'/parameter of mutant), when the value of the parameter of the mutant is less than that of the 'parent'.

Table 6. Kinetic parameters for hydrolysis of substrates by SHV-2A (Gly238Ser) and SHV-1

	Kinetic parameters					
	k_{cat} (/s)		K_M (μM)		k_{cat}/K_M (/μM/s)	
Compound	**SHV-1**	**SHV-2A (CF)**	**SHV-1**	**SHV-2A (CF)**	**SHV-1**	**SHV-2A (CF)**
1	250	150 (−0.67)	40	13.5 (−1.9)	6.3	11 (0.7)
2	19	29 (0.52)	42	8 (−4.3)	0.5	3.6 (6.2)
3	290	108 (−1.7)	220	25 (−8.1)	1.3	4.3 (2.3)
4	NM[a]	15 (NM)	>300	11.4 (<−25)	–	1.3 (>25)

[a]NM, not measurable.
Data taken from Lee *et al.* [25]. CF indicates the change factor, calculated as CF = (parameter of mutant/parameter of 'parent') – 1, when the value of the parameter of the mutant is greater than that of the 'parent', and as CF = 1 – (parameter of 'parent'/parameter of mutant), when the value of the parameter of the mutant is less than that of the 'parent'.

Table 7. Kinetic parameters for hydrolysis of substrates by TEM-10 (Arg164Ser, Glu240Lys) and TEM-12 (Arg164Ser)

	Kinetic parameters					
	V_{max} relative to hydrolysis of 3		K_M (μM)		V_{max}/K_M relative to hydrolysis of 3	
Compound	**TEM-12**	**TEM-10 (CF)**	**TEM-12**	**TEM-10 (CF)**	**TEM-12**	**TEM-10 (CF)**
1	1.8	1.7 (−0.1)	20	5.8 (−2.4)	90	293 (2.2)
4	0.04	0.09 (1.3)	94	46 (−1)	0.43	2.0 (3.7)
5	0.07	2.0 (28)	130	150 (0.15)	0.54	13 (23)
6	0.11	0.32 (1.9)	870	28 (−30)	0.13	11 (84)

Data taken from Queenan *et al.* [26]. CF indicates the change factor, calculated as CF = (parameter of mutant/parameter of 'parent') – 1, when the value of the parameter of the mutant is greater than that of the 'parent', and as CF = 1 – (parameter of 'parent'/parameter of mutant), when the value of the parameter of the mutant is less than that of the 'parent'.

protein (e.g., Glu104Lys) and it is uncommon among the thermally more stable TEM-2 derivatives. If it is not simply a natural polymorphism, then the selection may be for increased stability, regardless of the effects of other mutations.

Substitutions in the β3-strand

Mutations at three positions in the β3-strand, which forms one edge of the catalytic centre, have been observed.

Table 8. Kinetic parameters for hydrolysis of substrates by TEM-121 (Glu104Lys, Arg164Ser, Ala237Thr, Glu240Lys, Arg244Ser) and TEM-24 (Glu104Lys, Arg164Ser, Ala237Thr, Glu240Lys)

	Kinetic parameters					
	k_{cat} (/s)		K_M (μM)		k_{cat}/K_M (/mM/s)	
Compound	TEM-24	TEM-121 (CF)	TEM-24	TEM-121 (CF)	TEM-24	TEM-121 (CF)
1	20	25 (0.25)	9	15 (0.67)	2200	1700 (−0.29)
2	30	20 (−0.5)	30	420 (13)	1000	50 (−19)
3	90	70 (−0.28)	90	270 (2)	1000	260 (−2.7)
4	8.5	0.5 (−16)	25	430 (8.2)	340	10 (−33)
5	120	40 (−2)	180	150 (−0.2)	670	270 (−1.5)
6	16	6.5 (−1.5)	55	80 (0.6)	290	80 (−2.6)

Data taken from Poirel *et al.* [27]. CF indicates the change factor, calculated as CF = (parameter of mutant/parameter of 'parent') − 1, when the value of the parameter of the mutant is greater than that of the 'parent', and as CF = 1 − (parameter of 'parent'/parameter of mutant), when the value of the parameter of the mutant is less than that of the 'parent'.

Substitution of Ala237 for threonine or glycine is relatively uncommon (eight variants and only once, for threonine and glycine, respectively). The mutations increase both k_{cat} and K_M, but with a greater effect on the former parameter, resulting in an increase of k_{cat}/K_M for most substrates (Table 5).

Substitution of Gly238 for serine is very commonly encountered in the TEM and SHV series (32 variants and 22 variants, respectively). Substitution with aspartate (TEM-111) and alanine (SHV-13, -18 and -29) has also been observed. The mutations result in decreased turnover (k_{cat}) of simple substrates such as benzylpenicillin and cephaloridine, although the apparent affinity (K_M) increases, and therefore k_{cat}/K_M for these substrates is two- to three-fold greater in the mutants (Table 6). The effect on the hydrolysis of substrates with oxyimino acyl side chains is more marked, with significant increases in k_{cat} and k_{cat}/K_M. X-ray crystallography of enzymes with 238 substitutions shows a significant displacement in the 238–242 β-strand-turn segment, making the β-lactam-binding site more open [18]. In SHV-2, the β-strand is displaced by 1.6 Å with respect to its position in SHV-1 [18], while in TEM-52 [19] the loop between the β3 and β4 strands is moved by as much as 2.8 Å with respect to its position in TEM-1 and, in particular, the side chain of Glu240 is removed from the active site. The consequent widening of the active site could help to accommodate the larger oxyimino side chain, and the movement of Glu240 decreases the possibility of its interference with the substrate side chain.

Substitution of Glu240 for lysine is commonly encountered in the TEM and SHV series (22 variants and 15 variants, respectively). Substitution with arginine has also been observed (SHV-86). The substitution increases the apparent affinity (K_M) for most substrates, with little effect on turnover (k_{cat}), except for ceftazidime and aztreonam, for which the k_{cat} is increased more than three-fold (Table 7). These effects are not entirely consistent with the suggestion from crystallography that the side chain at position 240 causes steric hindrance to the binding of the substrate side chain, as the Glu240Lys substitution introduces a larger side chain. However, in the TEM series, the Glu240Lys substitution is always combined with substitutions at either Arg164 or Gly238, both of which affect the flexibility of the protein in the vicinity of Glu240 and could, therefore, ameliorate the effect of introducing a larger residue in this position. There are even three TEM variants with lysines at positions 104 and 240 that project from either side of the side chain-binding pocket of the active site (TEM-24, -46 and -121) and would potentially close the end of the active site, but these all have the Ω-loop mutation.

Substitutions at position 244

Substitutions at position 244 are associated with an inhibitor-resistant phenotype when present as a single substitution (e.g., in TEM-30, -31, -44, -51,

-54, -65) but are also found in combination with substitutions associated with an extended-spectrum phenotype (e.g., TEM-121). The substitutions replace arginine with a smaller amino-acid (serine, cysteine, histidine, leucine or glycine). The mutations decrease k_{cat} for most substrates and increase K_M for many, resulting in lower k_{cat}/K_M for all (Table 8). The decrease in reactivity of the enzyme is beneficial in slowing down the reaction with inhibitors such as clavulanic acid, altering the balance between acylation and rearrangement along the complex reaction pathways that ensue after acylation.

X-ray crystallography suggests that the substitution of arginine with a shorter amino-acid disrupts the hydrogen bond pattern that runs from this residue to Ser130 in the active site. The alterations in local structure and hydrogen-bonding may explain the decreased turnover and the lower apparent affinity of the mutant enzyme. Mutations at position 276 displace the side chain of Arg244 through altered interaction with its guanidine head group and have similar negative effects on the kinetics of the mutant enzyme, as does the direct replacement of Arg244 [20].

CONCLUSION

Class A β-lactamases appear to be well-ordered molecules in the ground-state but there is abundant evidence that they undergo several conformational changes induced by binding and reaction with substrates and mechanism-based inhibitors. These conformational changes result in branching reaction pathways that give a complex kinetic mechanism. It is probable that some of the mutations observed in clinical isolates affect the rate at which or extent to which these conformational changes occur and thus alter the partition between different branches of the pathways. This is an aspect that has yet to be studied in detail with any of the hundreds of variants that have been identified.

Furthermore, many substrates, and nearly all of the mechanism-based inhibitors, have the potential to undergo chemical rearrangement triggered by the attack of the enzyme on the β-lactam ring that can also lead to branched pathways and complex kinetics, which can be overlaid on the protein conformational change. Only the reactions with a few mechanism-based inhibitors have been studied, and a detailed description of the kinetic mechanism of any in terms of the structures of the intermediates and their rates of interconversion is lacking.

X-ray crystallography provides static pictures of several points in the reaction pathway and the preliminary basis for understanding the nature of the changes in protein structure consequent on the mutations. The relevance of any of the structures to the reactions occurring in solution is still uncertain, as none of the crystal structures provides an explanation for the protein conformational changes indicated by solution studies. Application of spectroscopic techniques such as nuclear magnetic resonance and Fourier-transform/infrared, which can provide time-resolved structural information, will be important in bridging this gap in our understanding.

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